Prothrombotic Gene Expression Profile in Vascular Smooth Muscle Cells of Human Saphenous Vein, but Not Internal Mammary Artery

S.K. Payeli, R. Latini, C. Gebhard, A. Patrignani, U. Wagner, T.F. Lüscher, F.C. Tanner

Background—The resistance of internal mammary artery (IMA) toward thrombotic occlusion and accelerated atherosclerosis is not well understood. This study analyzed gene expression profiles of vascular smooth muscle cells (VSMCs) from IMA versus saphenous vein (SV).

Methods and Results—54′675 probe sets were examined by Affymetrix microarrays. Thirty-one genes belonged to the coagulation system; 2 were differentially expressed, namely tissue factor (TF) and tissue-type plasminogen activator (tPA). TF was 3.1-fold lower in IMA than SV ($P=0.006$), whereas tPA was 9.0-fold higher ($P<0.001$). TF mRNA expression was lower in IMA than SV ($P<0.05$); tPA was higher ($P<0.001$). TF protein expression was 4.2 ± 0.5-fold lower in IMA than SV ($P<0.001$); tPA was 2.6 ± 0.4-fold higher ($P<0.01$). In IMA VSMC supernatant, TF protein and activity was lower ($P<0.05$), TFPI and tPA protein higher ($P<0.05$ and $P<0.005$), and clotting time of human plasma prolonged ($P<0.05$) as compared to SV. Migration to TF/FVIIa (10^{-9} mol/L) was 3-fold lower in IMA than SV ($P=0.01$); PAR-2 protein expression was similar ($P=$NS), PAR-2 blockade without effect ($P=$NS).

Conclusions—Among the genes of the coagulation system, TF and tPA are differentially expressed in VSMCs from IMA versus SV. This is consistent with protection of IMA from thrombus formation and vascular remodeling. (***Arterioscler Thromb Vasc Biol*. 2008;28:705-710**)

Key Words: bypass graft disease ■ tissue factor ■ tissue plasminogen activator ■ coagulation ■ migration

Coronary artery bypass grafting improves prognosis of patients with coronary artery disease.[1,2] Various factors predict graft patency, such as the surgical technique, cardiovascular risk factors, and low left ventricular ejection fraction.[3,4] In addition, the type of graft has a major influence on survival; indeed, patients with 2- or 3-vessel disease receiving an internal mammary artery (IMA) in addition to saphenous vein (SV) grafts exhibit higher long-term survival rates as compared to patients treated with veins only.[1,2] This difference is related to the occurrence of SV graft disease, an adaptive response of venous grafts leading to accelerated atherosclerosis, whereas the IMA is strikingly resistant toward such alterations.[5]

SV graft disease is determined by thrombosis, intimal hyperplasia, and accelerated atherosclerosis.[6] Thrombosis is the main cause for vein graft occlusion within the first months after bypass surgery, whereas the neointimal changes prevail at later stages. Even when conducted under optimized conditions, harvesting of SV grafts causes extensive endothelial disruption; indeed, more than 50% of the endothelial layer is denuded after preparation.[7] Loss of the endothelium activates coagulation via the exposure of tissue factor (TF), a key protease activator forming a catalytic complex with factor VIIa and thereby initiating coagulation, on vascular smooth muscle cells (VSMCs).[8] The important role of TF in the hemostatic activation phase early after bypass surgery is underscored by the observation that expression of TF is enhanced after coronary artery bypass grafting irrespective of whether an on-pump or off-pump procedure was performed.[9] To limit thrombus formation, vascular cells express fibrinolytic proteins such as tissue plasminogen activator (tPA), an enzyme mediating the conversion of plasminogen to plasmin. The endothelium is indeed a rich source of tPA; loss of the endothelial layer renders fibrinolysis dependent on tPA released from VSMCs.[10]

In the pathogenesis of bypass graft disease, thrombosis is interlinked with the development of intimal hyperplasia and accelerated atherosclerosis. VSMCs indeed migrate and proliferate in response to both coagulation factors and platelet-derived mediators.[11,12] TF/FVIIa is known to stimulate migration of VSMCs; accordingly, mice lacking the cytoplasmic domain of TF exhibit reduced neointima formation and vascular remodeling after femoral artery injury.[13] Hence, mediators primarily regulating thrombus formation in bypass

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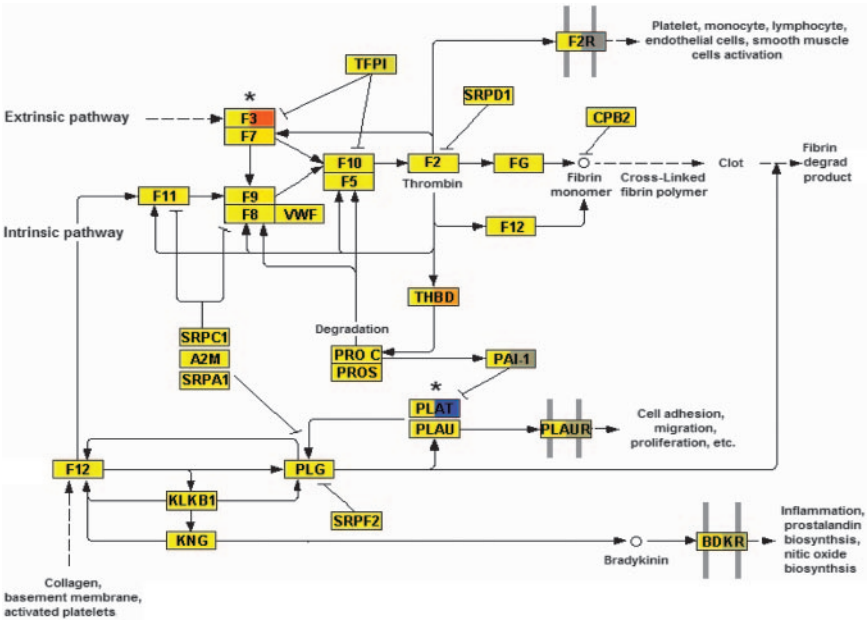

Figure 1. Expression of 54′675 probe sets is analyzed in vascular smooth muscle cells from internal mammary artery (IMA) and saphenous vein (SV) by the human genome U133 Plus 2.0 array. 31 genes are directly involved in coagulation; 2 of them are differentially expressed: tissue factor (F3) and tissue plasminogen activator (PLAT).

grafts may affect the development of later stages of bypass graft disease as well.

VSMCs are a heterogenous cell population, and different intrinsic properties of VSMCs from IMA versus SV seem to represent an important factor in the pathogenesis of bypass graft disease. VSMCs from IMA indeed exhibit lower contractility as well as lower proliferation and migration rates compared to cells from SV.[5,12,14] To improve our understanding of this heterogeneity, we compared the expression profile of genes involved in coagulation between VSMCs from IMA and SV segments retrieved during coronary artery bypass surgery using the Affymetrix microarray technology.

Materials and Methods

For the detailed Materials and Methods please see online supplement Materials and Methods at http://atvb.ahajournals.org.

VSMCs of IMA and SV were isolated from 9 patients undergoing coronary artery bypass grafting. VSMCs were cultured as described[14] and only compared if they originated from the same patient.

RNA was isolated using TRIZOL reagent. 15 μg of biotin-labeled cRNA samples were randomly fragmented at 94°C and hybridized to human genome U133 Plus 2.0 arrays. An Affymetrix gene chip scanner 3000 was used to measure fluorescent intensity. Values were always represented with respect to IMA VSMCs. Only genes exhibiting a more than 2-fold difference in expression were included for further analysis.

Real-time polymerase chain reaction (PCR) was applied to confirm microarray data as described.[15] Protein expression was determined by Western blot analysis as described[15]; alternatively, ELISA was used. The effect of VSMC supernatant on clotting time was determined using a S*tart* fibrometer after initiating coagulation by the addition of 50 μL calcium chloride. VSMC migration in response to TF/FVIIa complex was assessed in a 48-well modified Boyden chamber (Neuroprobe) as described.[12]

Results

Eight Genes Related to Blood Coagulation Are Differentially Expressed in VSMCs From IMA and SV

Gene expression profiles of VSMCs from IMA and SV segments obtained from 9 patients during coronary artery bypass surgery were assessed by Affymetrix human genome U133 Plus 2.0 arrays (54′675 probe sets). According to the Affymetrix database, 247 genes are related to blood coagulation; the relative expression levels of these genes are indicated along with the respective probability values (n=9) in the online supplement "Gene List" at http://atvb.ahajournals.org. Among the 247 genes, 8 genes (11 probe sets) were differentially expressed in VSMCs from IMA versus SV as defined by a more than 2-fold difference in expression level and statistical significance ($P<0.05$) in the online supplement Figure at http://atvb.ahajournals.org.

Two Genes Belonging to the Coagulation System Are Differentially Expressed in VSMCs From IMA and SV

The 247 genes related to blood coagulation were filtered by the Kyoto Encyclopedia of Genes and Genomes (KEGG; University of Tokyo) pathway database. This analysis indicated 31 genes belonging to the coagulation system (Figure 1). The gene expression profile of these 31 genes was compared between VSMCs from IMA and SV, which revealed 2 genes differentially expressed in VSMCs from IMA versus SV as defined by a more than 2-fold difference in expression level and statistical significance ($P<0.05$), namely tissue factor (TF; F3) and t-PA (tPA; PLAT). Expression of TF was 3.1-fold lower in VSMCs from IMA than SV ($P=0.006$), whereas that of tPA was 9.0-fold higher in IMA than SV ($P<0.001$). In contrast to tPA, expression of urokinase-type plasminogen activator (uPA) was not altered in VSMCs from SV as compared to IMA (n=9; P=NS).

Expression of tissue factor pathway inhibitor (TFPI), the physiological inhibitor of TF, and plasminogen activator inhibitor (PAI)-1, the endogenous antagonist of tPA, was assessed as well. Analysis of the 5 TFPI probe sets revealed very small differences in expression between IMA and SV; the average expression level was 1.2-fold lower in IMA as compared to SV. This difference was not significant in 4 of the 5 probe sets (n=9; P=NS), while reaching significance in 1 probe set (n=9;

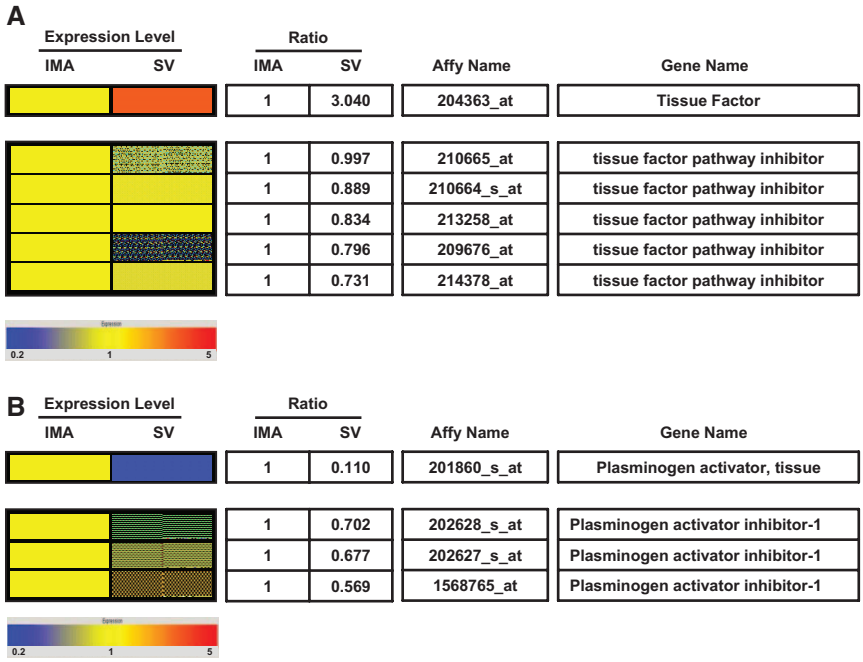

Figure 2. Expression profile of tissue factor (TF), t-PA (tPA), tissue factor pathway inhibitor (TFPI), and plasminogen activator inhibitor-1 (PAI-1) in vascular smooth muscle cells from internal mammary artery (IMA) and saphenous vein (SV).

$P<0.05$; Figure 2A). The 3 PAI-1 probe sets exhibited minor differences in mRNA expression between IMA and SV VSMCs; the average expression level in IMA was 1.5-fold higher than in SV, and this difference was significant in all the probe sets ($P<0.05$; Figure 2B).

Validation of Microarray Data at the mRNA and Protein Level

TF mRNA expression was analyzed by real-time PCR and observed to be 3.4±1.0-fold lower in VSMCs from IMA as compared to SV (n=4; $P<0.05$; Figure 3A, upper panel). The increase in TF mRNA expression after thrombin stimulation reached 2.6-fold in IMA and 1.9-fold in SV VSMCs as compared to basal level (n=4; $P<0.01$ for IMA and $P=0.01$ for SV). Real-time PCR also confirmed higher tPA mRNA expression in VSMCs from IMA as compared to SV (6.1-fold difference; n=4; $P<0.001$; Figure 3B, upper panel). Stimulation with thrombin did not affect the expression of tPA in VSMCs from IMA or SV (n=4; P=NS).

Western blot analysis for TF protein expression revealed a 4.2±0.5-fold lower TF protein expression in VSMCs from IMA as compared to SV (n=5; $P<0.001$; Figure 3A, middle panel). Similarly, cellular TF activity (cytoplasmic and membrane) was 1.4±1.9 pmoles in IMA and 29.0±3.1 in SV (n=5; $P<0.005$; Figure 3A, lower panel). The increase in TF protein expression after thrombin stimulation was comparable in VSMCs from IMA (1.8±0.4-fold; n=5) and SV (2.1±0.7-fold; n=5), and the expression level was 5.2±1.6-fold lower in IMA as compared to SV under these conditions (n=5; $P<0.05$). tPA protein levels were 2.6±0.4-fold higher in the supernatant of VSMCs from IMA as compared to SV (n=3; $P<0.01$; Figure 3B, middle panel), reaching 20.5±4.8 ng/mL in IMA and 3.3±1.8 ng/mL in SV (n=7; $P<0.01$; Figure 3B, lower panel). Stimulation with thrombin did not affect tPA levels in VSMCs from IMA and SV (n=3; P=NS).

Modulation of Coagulation by VSMCs From IMA and SV

The functional relevance of the different gene expression profile in VSMCs from IMA and SV was assessed. VSMCs were first serum-starved for 48 hours, and after this time period, cell supernatant was added to citrated human plasma followed by analysis of clotting time. When supernatant from

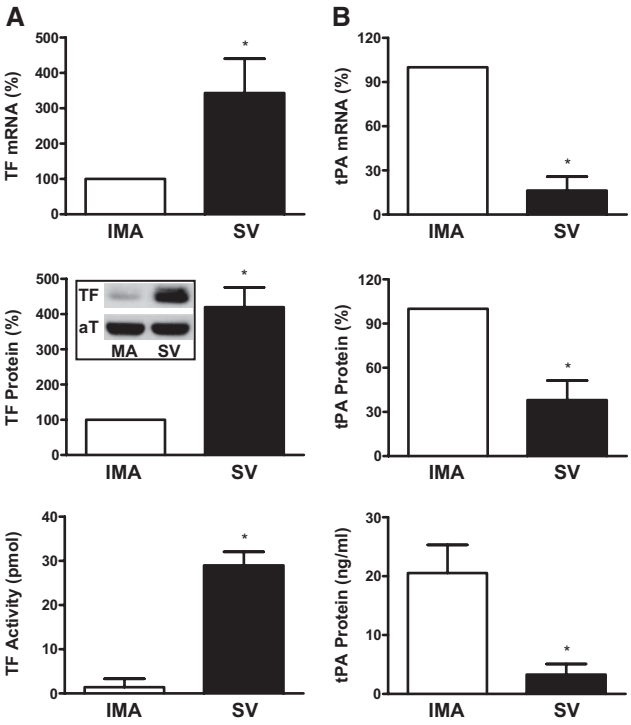

Figure 3. Validation of microarray data in vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV) at the mRNA level (upper panels) by real time-PCR and protein level (middle and lower panels) by Western blotting or ELISA.

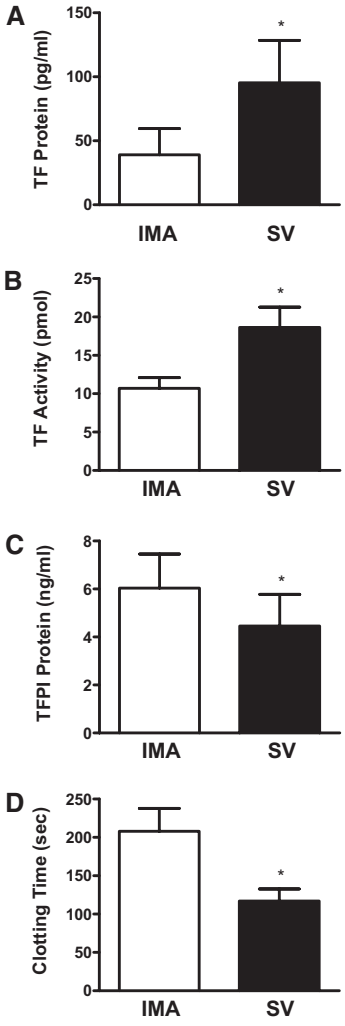

Figure 4. Modulation of clotting time of human plasma by supernatant of vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV). A, TF protein. B, TF activity. C, TFPI protein. D, Clotting time.

IMA was compared to SV, TF protein was lower (39.0±20.4 versus 95.3±33.1 pg/mL; n=5; $P<0.05$; Figure 4A) in an ELISA, TF activity was lower (10.7±1.4 versus 18.6±2.7 pmol; n=6; $P<0.05$; Figure 4B), TFPI protein was higher (6.0±1.4 versus 4.4±1.3 ng/mL; n=6; $P<0.05$; Figure 4C), and tPA protein was higher (Figure 3B). Consistent with these findings, clotting time was prolonged in the presence of supernatant from IMA (208±29 seconds) as compared to SV (117±16 seconds; n=4; $P<0.05$; Figure 4D).

Migration of VSMCs From IMA and SV

TF surface expression was slightly lower in VSMCs from IMA as compared to SV (Figure 5A). Similarly, TF surface activity was 1.4-fold lower in IMA (n=6; P=NS; Figure 5B). Migration in response to the TF/FVIIa complex (10^{-9} mol/L) was 3.0-fold lower in VSMCs from IMA as compared to SV (n=4; $P=0.01$; Figure 5C). PAR-2 protein expression was similar in both cell types (n=4; P=NS; data not shown). A PAR-2 cleavage blocking antibody did not affect the response of VSMCs to TF/VIIa (n=4; P=NS; data not shown).

Discussion

This study demonstrates that, among the genes of the coagulation system, only TF and tPA are differentially expressed in VSMCs from IMA versus SV. TF, the key protein for thrombus initiation, was expressed at a lower level in IMA VSMCs, whereas tPA, a major regulator of fibrinolysis, was expressed at a higher level in these cells. Consistently, conditioned media from IMA VSMCs induced a smaller reduction in clotting time of human plasma than media from SV. Moreover, IMA VSMCs responded to TF/FVIIa by a weaker migration than those from SV. These data demonstrate that IMA VSMCs exhibit intrinsic functional differences as compared to those from SV regarding the regulation of coagulation and vascular remodeling, and, although performed in vitro, offer an explanation for the protection of IMA from thrombosis and bypass graft disease. Although VSMCs were isolated from patients with coronary artery disease, neither IMA nor SV exhibited any atherosclerosis; hence, their properties reflect primary intrinsic differences and may be present in individuals without any atherosclerosis as well. Moreover, the properties of IMA do not seem to extend to other arteries, because it is unique in its resistance toward atherosclerosis, whereas veins in general may be similar to the SV.

As the major initiator of coagulation, TF plays an important role in the pathogenesis of thrombosis. Increased levels of TF antigen are detectable in atheroma of patients with acute coronary syndromes[16]; moreover, TF plasma levels are enhanced during and after coronary artery bypass surgery, suggesting that TF is involved in early graft occlusion.[17–19] This study demonstrates that VSMCs from IMA express less TF than those from SV at both the RNA and the protein level.[20,21] Although the difference in TF expression is smaller and may have less functional consequences than that in tPA expression, the lower TF expression in IMA VSMCs may protect this vessel from thrombotic occlusion if an endothelial erosion or denudation occurs, which is particularly important in the early postoperative phase. Indeed, IMA exhibits a dual protection from thrombus formation, as it cannot only be prepared with less endothelial damage, but its subendothelial gene expression profile is less thrombogenic than that of the SV, where endothelial damage during surgical preparation is extensive.

Tissue factor activity is counterbalanced by its endogenous inhibitor, TFPI. In human arteries, TFPI diminishes thrombogenicity of atherosclerotic plaques and reduces fibrin as well as platelet deposition.[22] TFPI release was higher in VSMCs from IMA than SV; hence, the lower TF expression in IMA is not counteracted by a parallel decrease in TFPI and therefore would be expected to represent a true protecting factor in IMA. Because endothelial cells are a major source of TFPI, they may modulate the balance of TF and TFPI; however, these cells are difficult to isolate in sufficient numbers from human bypass vessels.

Subacute or late occlusion of coronary artery bypass grafts occurs as a result of migration and proliferation of VSMCs leading to neointimal growth and the accelerated formation of atherosclerotic lesions.[6] Besides activating the coagulation cascade, TF is involved in regulating vascular remodelling. Indeed, TF is the receptor for FVIIa and as such mediates cellular responses like migration and proliferation of VSMCs[23–25]; consistently, low TF expression induces less

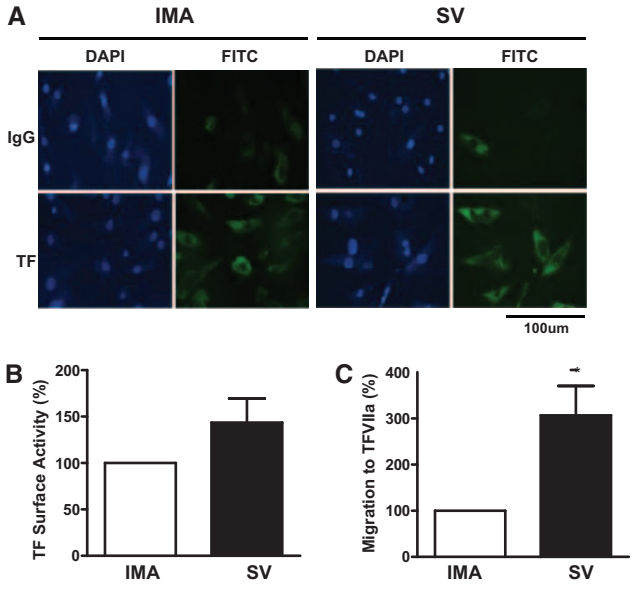

Figure 5. TF/FVIIa-induced migration of vascular smooth muscle cells (VSMCs) from internal mammary artery (IMA) and saphenous vein (SV). A, Immunofluorescence analysis of TF surface expression. B, TF surface activity. C, Migration to TF-FVIIa.

arterial remodeling in vivo. IMA VSMCs did not only exhibit lower TF expression, but also lower migration in response to TF, indicating that the IMA is protected from both thrombus formation and vascular remodeling. PAR-2 has been described to play a role in signaling the migratory response to TF/FVIIa.[24] However, VSMCs from IMA and SV exhibited similar PAR-2 protein expression, and a PAR-2 cleavage blocking antibody did not affect migration in response to TF/VIIa; hence, this receptor does not seem to regulate migration in response to TF/VIIa under our experimental conditions. The slightly lower TF surface expression and activity in IMA as compared to SV may only in part account for the lower migration of IMA VSMCs; hence, additional intrinsic differences in the regulation of migration may well exist. This interpretation is consistent with the observation that migration of IMA VSMCs is lower than that of SV in response to PDGF BB as well.[14] These properties may protect the IMA from remodeling and neointima formation and thereby promote the long-term success of IMA grafts.

Although it has been clearly demonstrated that the TF cytoplasmic domain regulates arterial remodeling in vivo,[13] it is still a matter of discussion which signal transduction events mediate this effect. Indeed, TF/VIIa was observed to activate the MAP kinases extracellular signal regulated kinase (ERK) and p38, the GTPase Rac1, and different Src family members.[25–27] The role of these mediators in regulating TF/VIIa-induced migration of VSMCs from human bypass vessels is not known and should be investigated in additional studies.

Antithrombotic mechanisms of vascular cells include the expression of tPA, a fibrinolytic enzyme mediating the conversion of plasminogen to plasmin. tPA indeed induces such an effective thrombolysis that its recombinant forms have several therapeutic indications.[28] This study reveals that tPA is expressed at a much higher level in VSMCs from IMA as compared to SV. VSMC supernatant from IMA indeed exerted a lower acceleration of clotting time than supernatant from SV, and this effect seems to be induced by a lower release of TF and a concomitant higher release of TFPI as well as tPA in IMA. These observations suggest a protective role of tPA in IMA. In line with this interpretation, adenoviral tPA gene transfer inhibits thrombus formation and promotes vessel patency in different models of vascular injury.[29,30] Hence, the higher tPA production in IMA VSMCs may be equally important for preventing thrombotic events and maintaining graft patency as its lower TF expression.

PAI-1 is a SERPIN that suppresses fibrinolysis by inhibiting the activity of tPA[31]; thus, excess PAI-1 activity would be expected to overcome the actions of tPA and increase the risk of thrombosis. However, in this study, PAI-1 gene expression was only slightly higher in VSMCs from IMA as compared to SV, indicating that the antithrombotic action of tPA in the IMA grafts is, if at all, to only a minor extent compensated by a concomitant increase in PAI-1 expression.

There is conflicting evidence on the functional role of tPA as a migration modulating factor. Some in vitro studies suggest that tPA stimulates VSMC migration, whereas others indicate that tPA induces migration only in the presence of plasminogen. More recent in vivo research reveals that tPA plays no role or has even a beneficial effect on neointima formation[32]; moreover, in vivo knockout models indicate that urokinase-type plasminogen activator (uPA), but not tPA, stimulates neointima formation.[33] No difference in expression of plasminogen or uPA was observed between VSMCs from IMA and SV, indicating that the effect elicited by the higher tPA production in IMA is not modulated by a concomitant difference in the expression of these fibrinolytic proteins. Further, fibrinolysis rather than facilitation of migration seems to represent the relevant action of tPA in bypass graft disease, as the IMA is resistant against both thrombotic occlusion and neointima formation, and this interpretation is consistent with in vivo studies on the role of tPA in vascular remodeling.[33] Moreover, because of intrinsic differences in the regulation of chemotaxis, VSMCs from IMA exhibit less migration than those from SV in response to mediators as different as PDGF BB and TF/FVIIa; hence, these cells would be expected to exhibit a weak migration even if tPA, despite of all the existing evidence, stimulated VSMC migration in bypass vessels.

In conclusion, this study suggests that the IMA is protected from thrombosis and neointima formation by an impaired TF expression in combination with an enhanced tPA production. Although these differences elucidate some properties of IMA, additional studies are required to fully understand the resistance of this vessel toward atherosclerosis. Nevertheless, these observations raise the question of whether a local genetic anticoagulant treatment should be considered in patients with venous bypass grafts in addition to systemic antiplatelet therapy. This question, however, remains to be answered in appropriately designed clinical trials.

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Disclosures

None.

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